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ABSTRACT

To date, few researchers have assessed the toxicities of Polycyclic Aromatic Hydrocarbons (PAH's) as mixtures, although that is how they commonly occur in the environment. The toxicities of 2,3, and 4 ring PAH's were determined for individual compounds. Synergistic antagonists or additive effects of mixtures of two, three, and four PAH compounds were assessed by comparing the toxicities of those mixtures to that of individual compounds. Microtox® mixture results were also compared to those predicted by the Σ PAH model. The toxicities of chrysene, pyrene, phenanthrene, fluorene, fluoranthrene, acenaphthalene, phenanthrene, and naphthalene were evaluated using the Microtox® acute and chronic toxicity assay which uses a bioluminescent bacteria, *Vibrio fisheri*, to measure toxicity. Results indicate that, as individual compounds, the acute toxicities of PAHs are inversely related to water solubility. The research also showed that mixtures can be synergistic, antagonistic, or additive. Chronic tests showed that PAHs with lower water solubility require greater concentrations than other PAHs to show a toxic response. Lastly, from the research, one can see that the additive concept for PAH mixtures is generally upheld.

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Assessment of Chronic and Acute Toxicity of Polycyclic Aromatic Hydrocarbons (PAHs) and PAH Mixtures

INTRODUCTION AND OBJECTIVES

Introduction: Literature review and background

PAHs

PAHs are multi-ring structures held together by stable carbon-carbon bonds that can be substituted with either nitrogen, sulfur, or oxygen (Figure 1) (Blumer, 1976). They occur in the environment when complex organic substances are exposed to high temperatures or pressure (Agency for Toxic Substances and Disease Registry, 1993). PAHs are common contaminants of hazardous waste sites and are present in aerial fallout of combustion products, industrial and sewage leakage, and the accidental disposal of petroleum products (Cerniglia, 1992). PAHs occur naturally in the environment due to oil seeps, surface run-off, and forest and prairie fires, but their concentrations can be elevated due to human activities (Chaundry, 1995).

Physiochemical characteristics, such as low water solubility and high octanol-water coefficients, affect the persistence of these compounds in the environment. PAHs are not very soluble, and their water solubility decreases with increasing molecular weight. PAHs have a high affinity for organic matter and organic containing particles (MacGillivery and Shiaris, 1995). PAHs therefore tend to associate with

sediment, where they partition to the organic fraction which can reduce their bioavailability. They are buried and persist until degraded, bioaccumulated, or removed by dredging (Cerniglia, 1992). There is therefore a direct relationship between PAH toxicity and bioavailability. PAH degradation is also influenced by such things as temperature, pH, nutrients, oxygen, and soil type.

PAHs consist of 2-7 benzene rings; PAHs with less than four benzene rings may be volatililized, photooxidized, oxidized, bioaccumulated, absorbed in the soil, leached or biodegraded by microorganisms (Cerniglia, 1992; Park, et al, 1990; Bossert and Bartha, 1986). PAHs with four or more benzene rings are less biodegradable, less mobile, and persist longer in environmental matrices. High molecular weight PAHs are desorbed slowly and therefore are less available for biological uptake. The half-lives of high molecular weight PAHs like benzo[a]pyrene are also longer than low molecular weight PAHs like naphthalene. These PAHs are recalcitrant and can persist in the environment for long periods of time (Cerniglia, 1992).

Recently, research has been conducted to test the degradability of higher molecular weight PAHs. A study by MacGillivry and Shiaris in 1993 found yeast to have the ability to degrade phenanthrene and benzo{a}anthracene (MacGillivery and Shiaris, 1993). It has also been found that *Mycobactium* sp. PYR-1 has the ability to mineralize fluoranthene, naphthalene, and pyrene in pure culture and also enhance mineralization of fluoranthene and pyrene in sediments containing native microorganisms (Cerniglia and Kelly, 1995)

The toxic, mutagenic, and carcinogenic nature of PAHs is a concern to both human and environmental health (Agency for Toxic Substance and Disease Registry, 1993). Exposure to PAHs proves to be a significant health risk to people living in the industrialized areas of the world. For example, PAH contamination ranges from 5ng/g of soil in undeveloped areas and $1.79x\ 10^6$ ng/g at an oil refinery (Cerniglia, 1992). The 1775 study by Pott found chimney sweeps to have a high incidence of cancer, presumably due to their exposure to PAHs (Agency for Toxic Substance and Disease Registry, 1993). In the 1930's Benzo{a}pyrene was isolated from coal tar, a constituent found in soot, and found to be carcinogenic. Concentrations of PAHs in coal tar can be orders of magnitude greater than those found in petroleum derived materials (Machado et al, 1993).

PAH exposure has not only been linked to several types of cancer but it is also involved in affecting the hematopoetic and the immune system. PAHs may affect the human fetus as well (Agency for Toxic Substance and Disease Registry, 1993) In 1993, Aspostoli et al. studied the effects on workers of prolonged exposure to cutting fluids; cutting fluids are mixtures containing various percentages of mineral oils and/or, more rarely, synthetic oils. These fluids are used mainly in mechanical engineering and the metal fabrication industry. It was concluded from this research that PAHs were mainly responsible for carcinogenic activity (skin and respiratory tumors) associated with the use of cutting fluid over time. PAHs, moreover, are listed on the EPA National Pollutant Priority List (Agency for Toxic Substances And Disease

Registry, 1993). Appropriate evaluation of potential adverse risk from PAH exposure is dependent on the appropriate toxicological assessment of PAHs.

Toxicity assessments using experimental animals have been performed for individual PAHs (Kwan, Dutka, and Liu, 1990). In the environment, however, PAHs are almost always found as compound mixtures. Mixtures exist in the sediment because sediment contamination usually involves the settling of many chemicals (Swartz et al, 1995). It has been found that PAH mixtures are sometimes more toxic than individual PAH toxicity. The combined stresses of multiple contaminants, or in this case PAHs, may cause environmental effects that would not be expected when PAHs are tested individually. PAH mixtures are more resistant to degradation and exhibit antagonistic, synergistic, or additive characteristics (Chaudry, 1995; Kelley and Cerniglia, 1995).

Mixtures that exhibit antagonistic characteristics are those that show a less toxic response than the sum of the individual compounds tested. Synergistic responses will occur when the mixture toxicity is greater than that of the individual compounds tested. Additive toxicity responses are those that have mixture toxicity equal to the sum of the individual compound toxicity. A 1990 study with benzo(a)pyrene (BAP) showed that synergistic toxicity responses occurred when BAP was tested in a binary mixture of polychlorinated aromatic hydrocarbons (Dutka and Kwan, 1982 and Donnelly at al, 1990).

Σ PAH Model

The Σ PAH Model was developed in 1995 by Dr. Robert Swartz to estimate the toxicity of PAH-contaminated sediments and relate mixture responses to individual

compound data. Before this, contaminated PAH sites were evaluated based on individual PAH toxicity. The Σ PAH model assumes that field collected mixtures exert a toxic stress that is due to the additive toxicity of the individual compounds present. It is therefore possible to predict PAH toxicity by calculating the toxic units of the individual compounds, in this case, using amphipod data.

Toxic units (TU) were calculated because they can be derived from all types of toxicity measurements and corrected for the solubilities of the compound. The TU is based on the solubility limits of the PAHs in the additivity model. It can be defined as the unit equal to the concentration of a chemical that kills 50%of test specimens in a toxicity test (Sprouge, 1970). TU were calculated by multiplying the PAH water solubility by the LC_{50} of the PAH for each individual compound. To date, 13 PAHs have been tested with promising results. The total number of toxic units of the PAHs (Σ TU) was calculated, assuming additivity, by adding the individual TUs of each compound (Swartz et al, 1995).

The Σ PAH model was verified by comparing mortality predicted by the model with the mortality observed in sediment toxicity tests in field-collected samples. It was found that the model accurately predicted the toxicity of PAH-contaminated sediments when PAHs were the principal contaminant. However, when a PAH was not the principal contaminant, significant differences between the predicited and the observed toxicity were observed (Swartz et al, 1995).

There is an 86.6% correspondence and no significant difference between predicted toxicity from the model and actual observed toxicity at PAH contaminated sites where PAHs are the dominant contaminant. This model also corresponds well with several

sediment quality guidelines such as: EPA Sediment Quality Criteria (EPAASQC) for the protection of benthic organisms, Apparent Effect Threshold (AET), State of Washington Cleanup Screening Levels (WACSL), State of Washington Sediment Quality Standards (WASQS), Effects range-Low (ERL), Effects range-high (EFH), and Screening Level Concentrations (SLC) (Swartz et al, 1995).

This approach is still being studied as a way to predict PAH mixture toxicity in sediment, and there are some uncertainty and limitations to it. For example, this model does not take into account the uncertainties between marine and estuarine amphipods in PAH sensitivity. The Σ PAH Model also does not acount for the potential effects of natural variables such as sediment grain size, total organic carbon, and redox potential (Swartz et al, 1995).

***Microtox®** - Acute*

Since it's development in 1979 by Beckman Instruments, the Microtox® assay has been used to determine and monitor soil and sediment, wastewater, surface water, and also ground water toxicity (Bulich, 1984; Somasundoram et al, 1990). Some state, and federal regulatory agencies use this system in screening tests to monitor environmental pollutants (Somasundoram et al, 1990). Microtox® is used by the City of Chattanooga to rank all the local industrial discharges in order of relative toxicity and in order of relative toxic impact on the City's wastewater treatment plant (Kurz, 1984). Microtox® has been recommended by The American Society for Testing Materials for biological testing of water leachates. The US EPA has recommended

Microtox® for testing treated effluents, for predicting land treatability of organic wastes and for bioassessment of waste disposal sites (Munkittrick et al, 1991). Taiwan has begun to use the Microtox® system to screen its industrial wastewaters (Hao et al, 1996). Microtox® is a toxicity test that has become one of the standard toxicity assays (Tarkpea et al, 1986).

Microtox® measures the decreased bioluminescence of the bacteria *Vibrio fischeri* in the presence of a toxic agent. The luciferase enzyme from this bacteria uses reduced flavin and oxygen to produce light (Chang et al, 1981). A photometer measures the decrease of light and gives a reading of the percent light loss, (Gamma). An Effect Concentration or EC_{50} is then produced by graphing the log of the Gamma response versus the log of the sample concentration. The EC_{50} is the chemical or environmental sample concentration causing a 50% decrease in the Microtox® Reagent light output under defined conditions of time and test temperature (Microbics, 1989). The decreased intensity of bioluminescence is proportional to the toxicity of specific chemicals or environmental samples. The smaller the EC_{50}, the more toxic the chemical or environmental sample.

Research has shown that the results of Microtox® are comparable to other assays. Canna-Michaelidou in 1993, compared Microtox® to a gas chromatography "fingerprint screening test". He compared the toxic effects of 7 mixtures of volatile organic pollutants (VOC). In doing so, he found that the Microtox® system was 140-

220 times more sensitive than the GC/FID for aromatic mixtures. This test was also found more sensitive for detecting regular petroleum and diesel toxicity.

Research has also been done comparing Microtox® to fish, *Daphnia*, and other microbial toxicity methods.

Microtox® vs. Fish. The 24-to-96-hr fish Lethal Concentration 50 (LC_{50}) acute bioassay is one of the most widely used acute toxicity tests. There has been extensive research showing that Microtox® is as sensitive as this test, especially when using the flathead minnow (Indorato et al, Curtis et al, 1982, E.V.S. Consultants, 1989, Bulich et al, 1980, Munkittrick et al, 1991).

In 1993, DeZwart and Sloof determined the toxicity of 15 chemicals by comparing the Microtox® acute assay to that of green algae, *Daphnia*, and fish. Their results showed the Microtox assay to be comparable to the other tests, especially the acute tests conducted with fish. Differences between the fish and Microtox® sensitivity were within one order of magnitude.

The City of Chattanooga has not only found the test to be more sensitive, but also less expensive and time consuming. Compared to the State of Tennessee, which averaged 10 or less bioassays a year using the LC_{50} fish toxicity test, Chattanooga conducted 160 tests using Microtox® in less than 6 months time (Kurz, 1984).

Microtox® vs. *Daphnia*. The comparative sensitivity of Microtox® and *Daphnia* is dependent on the compound tested. Microtox® is comparable to *Daphnia* toxicity tests, however the latter is often more sensitive (Calleja et al, 1986). For

example, *Daphnia* is more sensitive to ammonia, cyanide, hexachloroethane, pentadione, and sodium lauryl. Microtox®, on the other hand is more sensitive to chloroform, styrene and highly substituted organics (Munkittrick et al, 1991).

Microtox® vs. other Microbial toxicity methods. In 1981, Dutka and Kwan compared Microtox® to three other microbial toxicity tests: *Spirillum volutans, Pseudomas fluorescens,* and *Aeromonas hydrophila.* The Microtox® test proved to be the most sensitive to the chemicals tested (with the exception of Hg^{++}, Ni^{++}, Pb^{++}, and N-Nitrosodiethylamine). Microtox® was proven to be a more reliable and sensitive test when compared to the resazurin reduction (RR) method and the dissolved oxygen depletion (DOD) test (Greene et al, 1985). Microtox® also takes less time than other bioassays and has a statistical advantage since results are based on using over 10^5 bacteria. When McFeters tested the two organism Tchan procedure (algae and luminescent bacteria) with Microtox® he found Microtox® to be a more sensitive test .

Relative sensitivity to Microtox® has been compared to a variety of bioassay methods for the toxicity of chemicals, sediments, and complex effluents. When compared to fish and *Daphnia* studies, several researchers reached many of the same major conclusions. Munkittricks summarizes these conclusions as follows (Munkittrick et al , 1991):

- Organics - Microtox® is as sensitive as other assays for the measurement of pure organics. Microtox® is generally more sensitive to complex compounds such as

multichlorinated benzenes, phenols, and ethanols. The system is less sensitive to cyanide, chloroform, or phenol.

- Inorganics - Microtox® is not as sensitive as other tests to most inorganics . It is however, more sensitive to mercury, arsenic, and cobalt.
- Municipal Wastes - Microtox® is a favorable bioassay for monitoring municipal wastes.
- Industrial Wastes - Microtox® can also be used to screen for the relative toxicity of complex effluents.
- Sediment Contamination - The sensitivity of Microtox® varies with contaminant extraction technique (Munkittrick et al , 1991).

Microtox® not only has been used to test individual toxicants, but has also been used to test some mixtures. Many have found mixtures to be the sum of their toxic parts or as being additive (Sellers and Ram). However, it has been discovered over time that mixtures can react synergistically and antagonistically as well.

In Canna-Michaelidou's study of volatile organic pollutants, he found mixtures to show additive effects (Canna-Michaelidou,1993). Based on exposure time, and the proportion of chemical component, heavy metal mixtures were categorized as synergistic, antagonistic, or additive. The results showed that after a 45-minute exposure, Cd-Zn interacted synergistically, Cd-Ni interacted antagonistically, and Co-Ni and As-Pb interacted additively (Sellers and Ram). Walker studied the toxicity of zinc and pentachlorophol (PCP) in 1987 and determined that the interactions between the two were additive.

Dutka and Kwan (1981) also tested mixtures with Microtox®. They, like Canna-Michaelidou found that mixtures produced responses that were synergistic, antagonistic, or additive, based on the concentrations of the individual chemical and the incubation or contact time. For example, different concentrations of phenol were combined with the same concentration of sodium lauryl sulphate. Controls showed that phenol had a decreased toxic effect with increased incubation time; the toxic effect of sodium lauryl sulphate was stable over a 15-minute period. When the two were combined, however, there was an increase in toxic effect. The 20 ppm phenol combination showed an additive response, while 2.5 ppm phenol combination with 0.5 ppm of sodium lauryl sulfate showed a synergistic response. An antagonistic response was seen when high concentrations of Hg^{++}(0.075 ppm) were combined with Pb^{++}(5 ppm).

Microtox® is sensitive, rapid, and inexpensive relative to other assays (Ribo and Kaiser, 1987). The task of breeding test organisms is eliminated in this system. Assays that take two or three days using standard toxicity test can be conducted in five to thirty minutes with comparable results (De Zwart, 1983).

Microtox® -Chronic

The Chronic toxicity test is a new addition to the Microtox® assay system. Therefore, there has not been much published concerning this test. Chronic toxicity tests are used to predict adverse biological effects that result when an organism is exposed over its entire life cycle (Microbics, 1996). Like the acute test, the

Microtox® chronic toxicity test has been shown to be as sensitive or more sensitive than other chronic tests (Microbics, 1996). This test also takes less time than others, 22 hours.

This test uses the Model 500 Analyzer to detect light loss. To analyze the results of the Microtox® chronic toxicity test, the software system ToxCalc™, is used. It includes such statistical options as: a parametric hypothesis test, a non parametric hypothesis test, and point estimates can be used to determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC). The NOEC is the highest concentration tested that causes no statistically measurable effect on the test system. The LOEC, on the other hand, is the lowest concentration tested that causes a statistically measurable effect on the test system (Microbics, 1996).

Objectives

This research was designed to compare the toxicity of PAHs as individual compounds and as mixtures, as they are found in the environment. PAH mixture toxicity has not been studied as fully as that of individual compounds. The Microtox® toxicity test, both acute and chronic, were used to determine toxicity of PAHs and the interactions of the mixtures.

The specific objectives of this research project were to:

1. Assess the acute and chronic toxicity of PAHs individually and as mixtures using the Microtox® assay system.

2. To determine if the toxicity response of PAH mixtures is predictable based on data generated with individual compounds using the additive model (Swartz et al, 1995).

3. To evaluate differences between acute and chronic toxicity assay results.

MATERIALS AND METHODS

Chemicals

Eight PAHs were evaluated by the Microtox® bioassay: naphthalene, chrysene, pyrene, anthracene, fluoranthrene, fluorene, acenaphthene (Aldrich Chemical Co., Milwaukee, WI) and phenanthrene (Sigma Chemical Co., St. Louis, MO), (Figure 1).

PAH sample preparation

Solutions of individual PAHs were prepared by dissolving each PAH in two separate solvents, methanol and a 2% NaCl solution at their respective water solubility concentrations. For 2% NaCl solutions, saturated solutions of each individual PAH were prepared by first weighing the chemical to at least 1.5 times the standard solubility in 10 mL of distilled, deionized water. The saturated NaCl solutions were mixed well and allowed to settle. Supernatant was removed to make the sample mixture. Solutions of PAH and methanol were prepared by dissolving the weight of the chemical necessary to provide water solubility of the PAH in 10ml of methanol. Toxicity comparisons were made between NaCl solutions and methanol solutions for each individual PAH.

Microtox®- Acute toxicity

The Microtox acute toxicity test evaluates toxicity by comparing the luminescence of *Vibrio fischeri* alone to the luminescence in the presence of a particular chemical or environmental sample. This assay can be completed in 5 to 30 minutes. The Microtox® system includes a Microbics Model 500 Analyzer to measure

light intensity. This system is maintained at a temperature of 15+/- 0.1^{0}C. The bacteria (*Vibrio fischeri*), is the reagent used to detect toxicity. Diluent (2% NaCl) is used to provide osmotic stability for the bacteria. Reagent reconstitution solutions, which consists of high quality water, is used to reconstitute the bacteria. Bacteria are reconstituted, then allowed to stabilize for 15 minutes prior to chemical exposure. The software program (Microtox Release 7.0) is then used to calculate the EC_{50} of the chemical or environmental sample from changes in bacterial luminescence (Microbics Corporation , 1989). The EC_{50} is the reported percentage of sample that decreased bioluminescence of by 50%. The more toxic samples have a lower EC_{50} in this method because they required a more dilute sample of chemical to obtain a 50% response. Each sample was run in duplicate.

Individual PAH Testing

For PAHs dissolved in the 2% NaCl solution, 2.0 mL of 2% NaCl diluent was pipetted with PAH in the first cuvette. For individual PAHs dissolved in methanol, 10 ul of PAH with methanol was added to 2.0 ml of 2% dilient. Following addition of PAH with NaCl or PAH with methanol, 1ml of mixed diluent and PAH were added to the remaining cuvettes by performing three 1:2 serial dilutions. The last cuvette, which contained only diluent, was used as the control. 20.0 uL of reagent bacteria was pipetted into every cuvette after the computer software was set-up. After five minutes, the light measurements were read by the computer software, and the EC_{50} was calculated.

PAH Mixture Testing

Prior to toxicity testing of PAH mixtures, a 100ul sample of methanol was first evaluated for background toxicity. Dual PAH mixtures were made by adding the most toxic PAH solutions to those PAH solutions that were less toxic based on individual Compound results. In this case, four mixtures were tested: fluoranthrene-naphthalene, acenaphthalene-napthalene, fluorene-naphthalene and anthracene-naphthalene. Using the prior individual PAH solutions, 10ul of each desired PAH was added to make a mixture.

For triplicate and quadruplicate PAH mixtures, the dual PAH mixtures were combined with other PAHs based on toxicity differences. This experiment was designed to determine if the addition of another PAH either more toxic, less toxic or equal in toxicity change the toxicity response of the initial mixture?". PAHs were tested in mixtures using the same technique as for the individual PAH toxicity tests.

Twelve mixtures were tested (see table 2 and 3). To the first cuvette of each sample, 10 uL of each PAH mixture solution was added. After three 1:2 serial dilutions, parameters for the test were set. Reagent was added to each cuvette by using a 10.0 uL pipettor. Readings were completed by the Analyzer, and an EC_{50} was calculated for each sample.

Chronic toxicity

The Microtox Chronic Toxicity test also measures the inhibition of light production in luminescent bacteria when they are grown, through incubation, in the presence of a toxic chemical or environmental sample. This assay also uses the freeze-dried bacteria, (*Vibrio*

fischeri), as the reagent to detect toxicity. Along with the reagent, the chronic test calls for the use of culture medium. This medium contains salts and other nutrient to support growth of the bacteria and light production. The medium is also selective with high salt concentrations and low nutrients to prevent the growth of other bacteria. Activation solution is used to rehydrate the bacteria. It is composed of a 3.5% NaCl solution, which provides the bacteria with osmotic protection Reconstitution solution is also used in the Chronic toxicity test to reconstitute the test medium. The Model 500 Analyzer is used to measure light intensity, and the ToxCalc software program is used for the statistical analysis (Microbics, !996).

After 22 hours of incubation in a water bath (27^{0}C), control cells undergo divisions and complete induction of the luciferase systems. The system is able to measure the concentration of chemical which causes a specific EC_{50}. Those samples with toxic concentrations will show low light readings as well as inhibition of cell growth. A reading is positive when it has significantly lower light output than the control. The software program is then used to calculate the NOEC and LOEC.

Chronic toxicity testing

To begin this procedure, cuvettes were first set-up in cuvette supports to represent the medium control positive control, and the sample PAH. The first row of the block was designated sulphate for the medium control. The positive control was placed in the next four rows. PAH sample was also run in quadruplicate. Four samples could be run at the

same time. The Model 500 Analyzer was set to measure chronic samples, and the water bath was stabilized at 27°C.

Media Preparation

Media control was prepared by adding 36ml of room temperature Reconstitution Solution to a vial of medium and allowing the medium to dissolve. 500ul was then pipetted into cuvette 1-4 of every row. To the 5th cuvette of each row, 1ml of media was added.

Positive Control Preparation

Cuvettes in rows 2-5 of the cuvette supports used were designated for the positive control, $CuSO_4$. 10ul of $CuSO_4$ was added to each cuvette. These cuvettes now contained both media and the positive control.

Sample Preparation

The remaining cuvettes were used for the samples; four samples could be tested simultaneously. Naphthalene, acenaphthalene, chrysene, and anthracene were tested for chronic toxicity. A new vial of media was reconstituted. Along with the 1ml of media to the 5th cuvette of each row, 10ul of a PAH solution was added . The remaining cuvettes contained only 500ul of media.

Test Procedure

To a vial of test reagent (bacteria), 9ml of cooled activation solution (3^0C) was added and the reagent was allowed to dissolve. To each cuvette, reagent was then added. To cuvette 1-4 of each row, 30ul of reagent was pipetted. To the last cuvette in those rows, 60ul of reagent was pipetted. When this was complete, 1:2 serial dilutions were made of the media control, positive control, and samples by transferring 500ul from cuvette to cuvette and mixing three times after each transfer. The cuvettes were then incubated for 22 hours in a 27^0 C waterbath. After the incubation period, the Microbics software system, ToxCalc™ was set up and light readings were made. The system was then able to determine the LOEC and NOEC of the samples tested by conducting several statistical tests.

Σ PAH Model

The Σ PAH model was used to compare the following:

1. Σ TU from individual compounds measured with Microtox® - EC_{50} data from individual PAHs were converted to TU ($1/EC_{50}$) and compared to TU from amphipod data. Σ TU was calculated by adding the individual TUs of each PAH in a given mixture.
2. Measured TU of actual mixtures - EC_{50} data from actual mixtures were converted to TU. These values were then compared to the Σ TU of each mixture.

3. Literature Σ TU values based on amphpod data - These values were compared to the the measured TU of actual mixtures.

These relationships were studied to determine if mixtures were additive, as the model suggests, or if other mixture responses occurr.

RESULTS AND DISCUSSION

Because of the seriousness of PAHs in the environment, several sediment guidelines have been established. These guidelines however are based on individual compound toxicity and therefore do not address the problem of PAH toxicity completely. PAHs occur in the environment as a complex mixture of compounds. This then leads to what is called the "mixture paradox". The guidelines, which are derived from the individual PAH toxicity test, might underestimate the ecological effects of the PAH sample collected in the field if there are interactions between the individual compound. This problem was soon recognized and a new approach, the Σ PAH model was established to detect PAH mixture (Swartz,1995).

When the Σ TU from individual compounds measured with Microtox® (Table 1) was examined, data showed that there was a fundamental difference between the response pattern of amphipod data and Microtox® data. Results are of the same order of magnitude. However, measured TU shows naphthalene to be the most toxic with a TU of 16.67, while the predicted TU from the literature show pyrene to be the most toxic with a TU of 10.00.

Individual PAH Acute Toxicity Tests

The first experiment was to test the toxicity of the individual PAHs (Fgure 2) and relate toxicity results to published values of water solubility (Figure 3, Table 1). EC_{50} values were converted to toxicity units or TU because they allow for comparison of compounds of different solubility. The larger the TU, the larger the PAH toxicity. The

less soluble the PAH, the more toxic that PAH. This can be seen with acenaphthalene and fluoranthrene. This can be seen in figure three, which is in toxic units. Acenaphthene has a water solubility of 3.9 mg/L and an EC_{50} of 1.23% (TU of 0.81) . Although this PAH is quite water soluble, it is not very toxic. Fluoranthrene is not very soluble, 0.27 mg/L , but it is more toxic with an EC_{50} of 0.23% (TU of 4.35). Acenapthene is composed of two benzene rings, while fluoranthene is composed of three. Neff studied the acute toxicity of aromatic hydrocarbons to freshwater and marine animals; it was fond that this trend in solubility and toxicity was upheld in other work as well (Neff, 1979) . A major exception is seen however to the solubility/toxicity trend in napthalene, which is the most soluble in water and is also the most toxic compound with an EC_{50} of 0.06%. There however is no available information to explain this occurrence. It is possible that naphthalene is soluble enough to have a sufficient concentration to exert toxicity. Another reason could be that molecules are able to pass the menbrane faster or to a larger extent than other PAHs .

PAH mixtures

Mixures of two, three, and four PAHs were tested for acute toxicity. The toxicity of these mixtures were characterized as additive if the actual measured TU was within a factor of two of the predicted value obtained by adding the individual compound toxicity units (see Table 1 and 2). Antagonistic responses were those that showed a measured TU of less than two times the predicted TU. Synergistic responses showed measured TU of two times more than the predicted TU. A factor of two was used because there was

insufficient data to calculate statistical variance. Varaibility between data was such that the differences of a factor of two were thought not to be significantly different.

Dual Mixtures

From the acute toxicity tests of the dual mixtures (Table 2), three mixtures proved to be additive: fluorene-naphthalene, acenaphthene-naphthlene and anthracene-naphthalene. The mixures of acenaphthalene-phenanthrene and fluoranthene-naphthalene were antagonistic. All but one of these PAH mixtures contained naphthalene, which is the most toxic and soluble.

Other Mixtures

Table 3 shows the results of three and four component mixtures. There was only one synergistic mixture among them. This mixure contains acenaphthlene and phenanthrene, which proved to be antagonistic in the dual tests as well. The non-toxic and very insoluble chrysene was able to change the solubility of the mixture and make it more toxic. The mixtures shown to be antagonistic all contained naphthalene. Naphthalene is the most toxic and most water soluble PAH tested. The addition of this PAH to mixtures may have given a less than additive responses because of its tendencey to sorb to the other compounds or to the container wall coated with the other PAH compounds of the mixture. This action would reduce the soluble concentration of naphthalene considerably and give a less toxic effect than that predicted by additivity. This was not seen with the two

component mixtures containing naphthalene. The overall amount of the PAH may not have been sufficient to provide the sorptive surface.

Σ PAH Model

1. Measured TU of actual mixtures (Table 3) - Most of these mixtures were additive. Those that showed less than additive (antagonistic) responses involved naphthalene. Again, naphthalene may be sorbing to other PAHs or to the walls of the container, making the concentration less in solution and therefore less toxic than predicted. One synergistic mixture was shown. This mixture contains chrysene, which is the least soluble and least toxic compound tested with the acute toxicity test. The addition of this compound to the mixture lead to greatly increased toxicity. Reasons for this are not understood, but warrants additional study.
2. Literature TU values are based on amphipod data (Table 3) - The comparison of Microtox® TU data to amphipod TU data does support the additivity concept for mixtures. However, there are differences between the two. The Σ PAH Model is based on PAH sediment data, while the PAHs used for the Microtox® acute tests were pure compounds. The model also is based on amphipod data, while Microtox® is based on bacteria data. Amphipods are smaller and have more surface area. Thier detox systems and organs may be able to store compounds in their lipids. Amphipod bioassays have been conducted extensively. However, while their results are comparable to other tests, they are not as sensitive as fish LC_{50} data when comparing

aquatic invertebrate bioassays (Sprogue, 1970; De Zwart and Sloof; 1983 Indorato et al, Curtis et al, 1982)

Many researchers have found mixtures of chemicals and toxicants to be additive, (Canna-Michaelidou, 1993; Seller and Ram; Swartz et al, 1995 and Dutka and Kwan, 1981) as well as antagonistic and synergistic (Donelly, 1990; Canna-Michaelidou, 1993; Seller and Ram; and Dutka and Kwan, 1981). For example, Dutka and Kwan tested several chemicals, Hg^{++}, Zn^{++}, Cu^{++}, Pb^{++}, Ni^{++}, naphthanol, phenol, sodium lauryl sulfate and 3,5 dichlorophrnol, in mixtures using several bioassays including Microtox®. It was found that mixture responses can be affected by exposure time and the amount of the chemical component use. In most cases, even though there were differences between the toxicity patterns within tests, the lower concentrations of some toxicants in combination had toxicity effects greater than higher individual toxicant concentrations. It was also discovered that a chemical, in one mixture may not have the same effect in another. Chemicals are able to inhibit the toxicity of another chemical in a mixture while competing for cationic sites, explaining why some mixtures do exhibit antagonistic responses (Dutka and Kwan, 1981). Synergistic responses also occur when mixtures are tested, however there is no known mechanism to explain this occurance.

Chronic toxicity

Several PAHs were tested using the Microtox® Chronic Toxicity test (Table 4). A positive control of copper sulfate, methanol, naphthalene, acenaphthalene, chrysene, and anthracene were tested. As can be seen from table 4, chrysene and anthracene have the

highest LOEC among the PAHs tested, both 100 ppb. This means it takes a higher concentration of these specific PAHs to have a toxic response. The concentration of chrysene tested proved to be non toxic in the acute test; this corresponds well with the chronic data of having to use an increased amount to show a toxic response. Anthracene is one of the more toxic PAHs tested with the acute toxicity test, however it shows low chronic results. The two toxicity tests in this case do not correlate. Moreover, chrysene and anthracene are the two least soluble PAHs tested. The data shows that there is an inverse relationship between the LOEC of a PAH and its water solubility. The lower the water solubility, the higher the LOEC.

CONCLUSION

PAHs have been in existence since the beginning of time (Chaundry, 1995). Even though they can be produced naturally in the environment, man has a pivotal role in increasing their abundance by the development, consumption, and combustion processes associated with the production of consumer goods. Thus, PAHs are a major concern and are the topic of many laboratory studies. Microtox® has been studied extensively over the years, and has been used as a screening and monitoring tool. It has proven to be reliable, fast and also inexpensive. Therefore, to study the relationship of PAHs and PAH mixtures in a fast and efficient manner, the Microtox® acute and chronic toxicity tests were used.

In conclusion, it was found through this research project that :

- Generally, with the exception of naphthlene, individual PAH toxicity is the inverse of the water solubility. The greater the water solubility, the less toxic the PAH.
- The research also shows the relationships between PAH mixtures. Mixtures can be either synergistic, where the individual PAH toxicity is less than that of the combined PAHs, antagonistic, where the individual PAH toxicity is more than the combined PAHs, or additive, where the sum of the individual PAH and the mixture have the same toxicity.
- Chronic toxicity tests with the Microtox system showed the concentration (ppb) of the PAH needed to be inhibit growth. Unlike the acute test, this test does not give a

toxicity value. One may be able to assume from the data presented that those PAHs with lower water solubility require greater concentration than other PAHs to show a chronic toxic response. If the chronic toxicity is known for a specific mixture, remediation techniques can be evaluated according to known water solubilities.

- When observing the results of the Σ PAH Model to Microtox® measured values one can see that (1) generally, the additive concept for PAH mixtures is upheld. (2) Microtox® toxicity data of mixtures is suppprted by other literature. (3) When a mixture was not additive, it was in most cases less toxic than predicited by individual compound data. This would provide a margin of safety from a regulatory perspective.

REFERENCES

Agency For Toxic Substances And Disease Registry. 1993. Polycyclic aromatic hydrocarbon toxicity. Am. Fam. Physician. **47**:623-628

Apostoli, P., Crippa, M., Fracasso, M.E., Cottica, D. and Alessio L. 1993. Increase in the polycyclic aromatic hydrocarbon content and mutagenicity in a cutting fluid as a consequence of its use. Int. Arch. Occup. Environ. Health. **64**:473-477.

Blumer, M. 1976. Polycyclic aromatic compounds in nature. Sci. Am. **234**:34-45

Bossert, I.G. and Bartha R. 1986. Structure-Biodegradability of polycyclic aromatic hydrocarbons in soil. Bull. Environm. Contam. Toxicol. **37**:490-495.

Bulich, A.A. 1984. Micrtox- a bacerial toxicity test with several evironmental applications. In: D.L. Liu and B.J. Dutka(ed.) Toxicity Screening using Bacterial Systems. Marcel Dekker, New York. pp. 55-64.

Bulich, A.A., and Isenberg D.L. 1981. Use of luminescent bacteria system for the rapid assessment of aquatic toxicity. ISA Transactions. **20**:29-33.

Calleja, A., Baldasano, J.M., and Mulet, A. 1986. Toxicity analysis of leachates from hazardous wastes via Microtox and *Daphnia magna*. Toxicity Assessment: An International Quarterly. **1**:73-83Proceduress.

Canna-Michaelidou, S. 1993. Microtox™ response to volatile organic pollutants implementation of the test in invertigating accidental or deliberate watersupply pollution. The Science of the Total Environment. Supplemnet. pp.969-976.

Cerniglia,C.A. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodrgradation. **3**:351-368.

Cerniglia.C.A. and Kelly,I. 1995. Degradation of a mixture of high-weight polycyclic aromatic hydrocarbons by a Mycobacterium strain PYR-1 Jour. Soil Contam. 4:77-91.

Chang, J.C., Taylor P.B., and Leach F.R. 1981. Use of Microtox assay system for environmental samples. Bull. Envirn. Contam. Toxicol. **26**:150-156.

De Zwart, D. and Slooff W. 1983. The Microtox as an alternative assay in the acute toxicity assessment of water pollutants. Aquatic Toxicology. **4**:129-138.

Donnelly, K.C.,Brown, K.W., AndersonC.S., Barbee, G.C. and Safe, S.H. 1990. Metabolism and bacterial mutagenicity of binary mixtures of benzo{a}pyrene and polychlorinated aromatic hydrocarbons. Environ. Mol. Mutagen. **16**:238-245.

Dutka, B.J . and Kwan, K.K. 1981. Comparison of three microbial toxicity screening tests with the Microtox® test. Bull. Environ. Contam. Toxicol. **27**:753-755.

Green, J.C., Miller, W.E., Debacon, M.K., Long, M.A., and Bartels, C.L. 1985. A comparison of three microbial assay procedures for measuring toxicity of chemical residues. Arch. Environ. Contam. Toxicol. **14**:659-667.

Hao, O.J., Shin, C., Lin, C., Jeng, F., and Chen, Z. 1996. Use of Microtox tests for screening industrial wastewaters. Wat. Sci. Tech. **10**:43-50.

Kurz, G.E., Leslie, N., and Henderson, J. A rapid industrial waste screening method. Presented at : The 39th Purdue Industrial Waste Conference.

Lebsack, M.E., Anderson, A.D., DeGraeve, G.M., and Bergman, H.L. 1981. Comparison of bacterial luminescence and fish bioassay results for fossil-fuel process waters and phenolic constituents. Aquatic Toxicology and Hazard Assessment: Fourth Conference. ASTM STP 737. D.R. Branson and K.L.Dickson, Eds., American Society for testing and Materials. pp.348-356.

MacGillivay, A.R. and Shiaris, M.P. 1993. Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. App. Environ. Biol. **59**: 1613-1618.

MacGillivay, A.R. and Shiaris, M.P. 1995. Microbial ecology of polycyclic aromatic hydrocarbon (PAH) degradation in coastal sediments. In: Caudry, G.R. (ed.) Biological Degradation and Bioremediation of Toxic Chemicals. Chapman and Hill, London. pp. 125-141.

McFeters, G.A., Bond, P.J., and Olson, S.B. 1983. A comparison of microbial bioassays for the detection of aquatic toxicants. Water Res **17**:1757-1762.

Microbics. 1989-1992. How to run toxicity tests using the Microbics Model 500. Microbics Corporation. Carlsbad, California.

Microbics. 1996. Microtox chronic toxicity test. Microbics Corporation. Carlsbad, California.

Munkittrick,K.R., Power,E.A., and Sergy,G.A. 1991. The relative sensitivity of Microtox®, daphnid, rainbow trout, and flathead minnow lethality tests. Environ. Tox. Water Qual.: An Internat. Jour.

Neff, J.M. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment: Sources, fate and biological effects. London: Applied Science Publishers LTD pp. 198-204.

Park, K.S., Sims, R.C., Dupont, R.R., Douchette, W.J., and J..E.Matthews. 1990. Fate of PAH compounds in soiltypes: influence of volatilization, abotic loss and biological activity. Environ. Toxical. and Chem. **9**:187-195.

Ribo,J.M. and K.L.E, Kaiser. 1987. Photobacterium Phosphoreum toxicity Bioassay. 1. Test procedures ans application. Toxicity Assessement: An International Quarterly **2**:305-323.

Scheepers, P.T.J. and R. Bos. 1992. Combustion od diesel fuel from toxicological perspective. Int. Arch. Environ. Health. **64**:163-177.

Sellers, K.E. and N.M.Ram. Toxicity assesment of binary metal mixtures using bioluminuous material.

Spargue, J.B. 1970. Measurment of pollutant toxicity to fish: 2.Utilization and applying bioassay results. Water Res. **4**:3-32.

Swartz, R.C., et al. 1995. ΣPAH : A model to predict the toxicity of polynuclear aromati hydrocarbon mixtures in field collected sediments. Environ. Toxical and Chem. **14**:1977-1987.

Walker, J.D. 1987. Effects of chemicals on microorganisms. Jour. WPCF. **59:** 614-615.

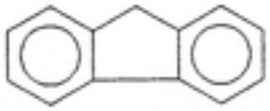
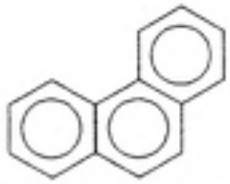
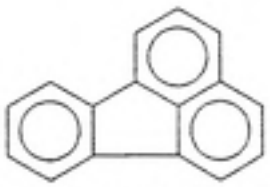
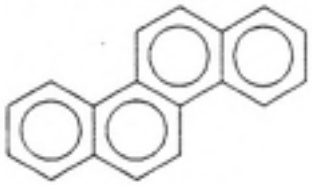
PAH	W_{SOL} (mg/l)	$W_{SOL-2\%NaCL}$ (mg/l)	K_{OW}
 NAPHTHALE (NAP)	31.7	27.7	3.37
 ACENAPTHENE (ACE)	3.9	3.5	4.33
 FLUORENE (FLU)	1.98	1.87	4.18
 PHENANTHRENE (PHEN)	1.3	1.2	4.46
 FLUORANTHENE (FLURA)	0.27	0.26	4.78
 PYRENE (PYR)	0.14	0.14	4.90
 ANTHRACENE (ANT)	0.07	0.06	4.45
 CHRYSENE (CHY)	0.002	0.002	5.49

Figure 1- PAH Structure, Water Solubility and Log Kow

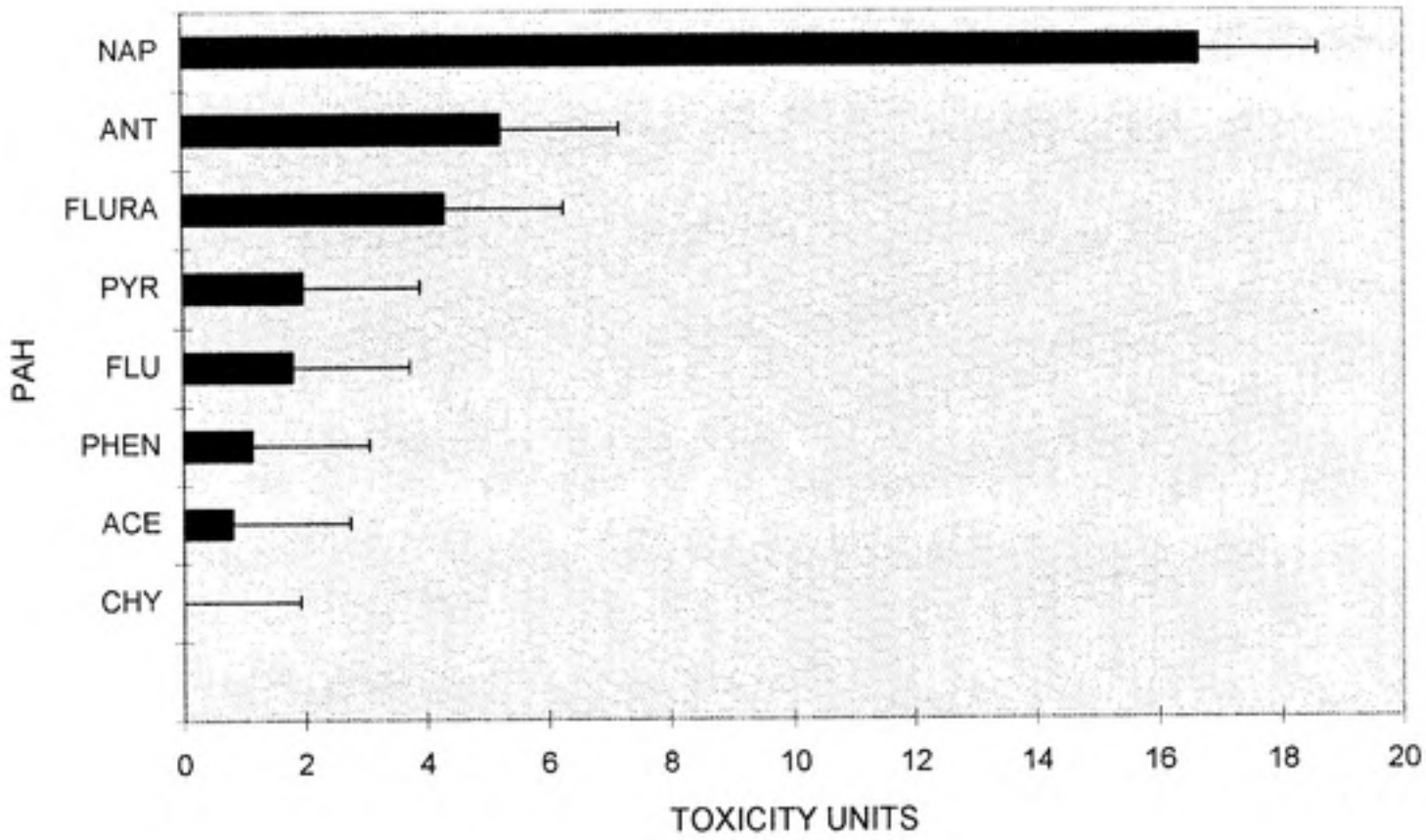


Figure 2--Individual PAH toxicity expressed in Toxic Units

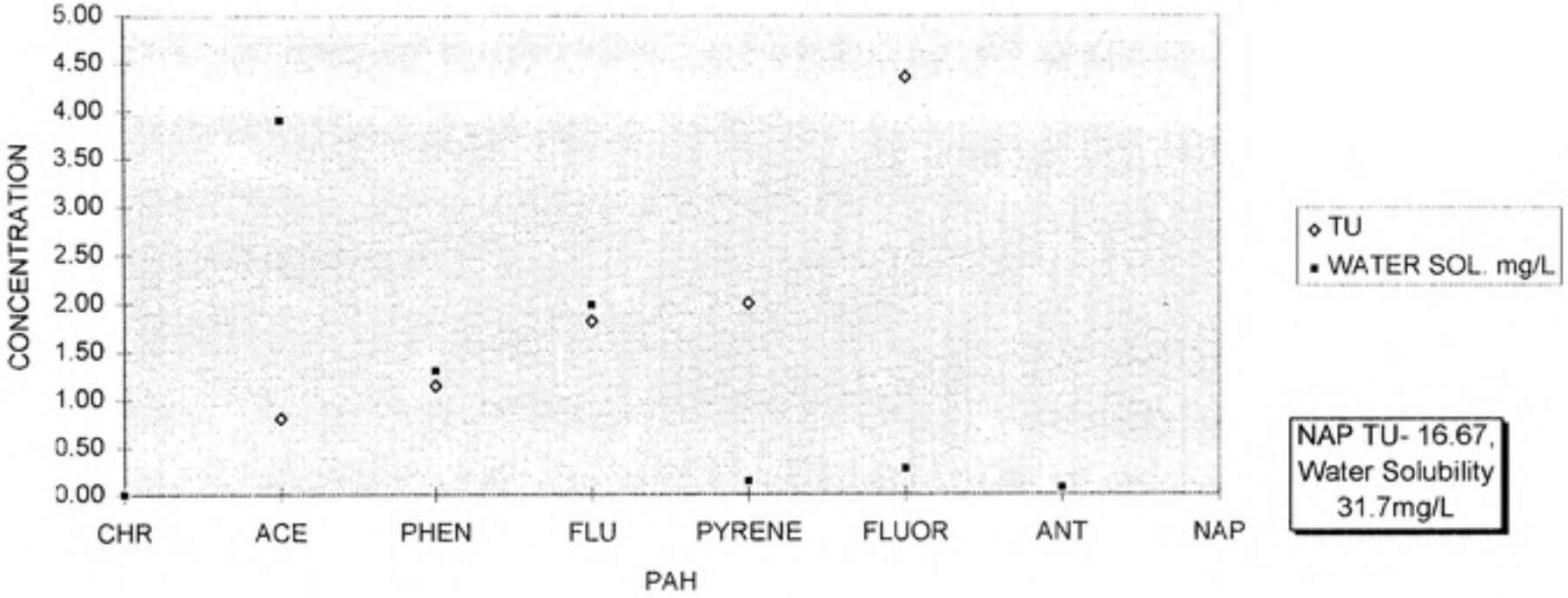


Figure 3 -- PAH Toxicity vs. Water solubility

Table 1--PAH TOXICITY UNITS OF INDIVIDUAL COMPOUNDS

PAH	WATER SOLUBI_ITY (mg/L)	EC50 (%)	MEASURED TU*	10-d LC50 (mg/L) TU**
CHRYSENE	0.002	NT	0.00	0.33
ACENAPTHENE	3.9	1.23	0.26	7.96
PHENANTHRENE	1.3	0.87	0.77	5.42
FLUORENE	1.98	0.55	0.51	7.33
PYRENE	0.14	0.5	7.14	10.00
FLUORANTHRENE	0.27	0.23	3.70	9.31
ANTRACENE	0.07	0.19	14.29	0.39
NAPTHLENE	31.7	0.06	16.67	9.06

* Based on Microtox data
**Based on literature

Table2--Dual Mixtures

DUAL MIXTURES	PREDICTED TU*	EC50 2%	MEASURED TU**	RESPONSE
ACE - NAP	17.48	0.03	33.33	ADDITIVE
FLU - NAP	18.48	0.04	25.00	ADDITIVE
ANT - NAP	21.93	0.03	33.33	ADDITIVE
ACE-PHE	6.23	NT	0.00	ANTAGONISTIC
FLUAN-NAP	17.67	0.44	2.27	ANTAGONISTIC

*Based on Microtox data of individual compounds

**Based on Microtox data of mixtures

Table 3--PAH TOXICITY UNITS OF MIXTURES

PAH MIXTURE	MEASURED SIGMA TU*	MEASURED TU**	PERDICTED SIGMA TU***	RESPONSE
CHY-AN-PYR	0.69	0.90	10.72	ADDITIVE
CHY-PYR-FLU	3.82	2.00	13.71	ADDITIVE
FLAUN-NAP	21.01	2.27	17.67	ANTAGONISTIC
FLUAN-NAP-PYR	21.30	2.50	25.29	ANTAGONISTIC
FLU-ACE-PYR	4.63	3.03	13.38	ADDITIVE
NAP-PYR-PHEN	20.48	3.33	22.69	ANTAGONISTIC
FLU-ACE-NAP	19.30	4.55	22.06	ANTAGONISTIC
FLUAN-FLU-PHEN-NAP	17.36	5.00	19.78	ANTAGONISTIC
CHY-PHEN-ACE	1.96	10.00	17.02	SYNERGISTIC
FLURA-PHEN-ACE	6.31	12.50	16.39	ADDITIVE
FLURA-FLU-PHEN	7.32	12.50	24.35	ADDITIVE
NAP-FLURA-ANT	26.28	20.00	26.39	ADDITIVE
FLU-NAP	16.48	25.00	18.37	ADDITIVE
ACE-NAP	17.48	33.33	29.78	ADDITIVE
FLURA-ACE-NAP	21.83	33.33	26.33	ADDITIVE
ANT-NAP	21.93	33.33	9.45	ADDITIVE
ACE-PHE	6.23	NT	13.37	ANTAGONISTIC

* Based on Microtox data of individual PAHs
**Based on Microtox mixture data
***Based on literature
NT=NOT TOXIC

Table 4--PAH CHRONIC TOXICITY

SAMPLE	LOEC (ppb)
NAPHTHALENE	50
ACENAPHTHALENE	50
CHRYSENE	100
ANTHRACENE	100
METHANOL	100
COPPER SULFATE	100

LOEC-Lowest observed effect concentration